TITLE OF THE INVENTION
INHIBITORS OF CORONAVIRUS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims benefit of U.S. provisional application 60/463,100 filed April 14, 2003, U.S. provisional application 60/467,190 filed April 30, 2003, U.S. provisional application 60/478,860 filed June 16, 2003, U.S. provisional application 60/479,429 filed June 18, 2003, and U.S. provisional application 60/479,430 filed June 18, 2003. All the applications are incorporated by reference.

BACKGROUND OF THE INVENTION

Coronaviruses are nonsegmented, s/s plus strand, enveloped RNA viruses that infect a variety of mammals and birds. In humans, they cause respiratory infections, enteric infections and possibly neurological conditions. Typically, infection results in a mild, self-limited condition, such as a cold or upset stomach. Recently, there have been reports that severe acute respiratory syndrome (SARS) is caused by a coronavirus, SARS-CoV. Although most SARS victims recover, the World Health Organization estimates that SARS is fatal in about 4% of cases, particularly in individuals with an underlying condition, such as a weakened immune response, diabetes or heart disease.

Coronavirus envelope protein generally carries two glycoproteins, M or membrane glycoprotein and S or spike protein. S protein is involved in receptor binding and cell fusion and is comprised of two subunits: the N-terminal subunit, called S1, which forms the knob-like component of the spike and the C-terminal subunit, called S2, which forms the stem of the spike. Little is known about the mechanism by which coronaviruses enter cells and presently there are no antiviral drugs consistently successful in treating any coronavirus infection, including SARS, or vaccines available to prevent infection.

The references cited herein are not admitted to be prior art to the claimed invention.

SUMMARY OF THE INVENTION

This invention relates to inhibitors of coronaviruses, such as SARS-CoV; methods of identifying inhibitors of coronaviruses; methods and compositions useful for therapy (treatment, or prevention) of coronavirus infections, including methods and compositions for therapy of severe acute respiratory syndrome (SARS); and compositions for use as vaccines and immunogens and methods in which they are used to vaccinate or immunize individuals against coronaviruses, including vaccines and immunogens for use in vaccinating or immunizing individuals against SARS.

In particular embodiments, the present invention relates to C-peptides, N-peptides, Five-Helix proteins, Heptad Repeat 2 (HR2) peptides, and Heptad Repeat 1 (HR1) coiled-coil peptides, all of which target HR1 or HR2 regions of the coronavirus Spike protein to inhibit coronavirus replication. They are for use as inhibitors of coronaviruses in therapy (treatment, or prevention) of coronavirus infection, including for use as vaccines or immunogens. The exact HR1 and HR2 regions may differ from the proposed regions. However, the proposed regions provided herein are sufficient to enable the design of the inhibitors.

Reference to "comprising" and "comprises" throughout the claim indicates additional groups may be present. For example, a C-peptide comprising about 18 amino acids of SEQ ID NO: 1 may have additional groups such as amino acid regions from SEQ ID NO: 1 or other types of groups.

Reference to "peptide" in a C-peptide and N-peptide indicates amino acids joined together by a peptide bond and salt forms. The amino acids may be naturally occurring amino acids or non-naturally occurring amino acids. A non-naturally occurring amino acid (also referred to as a "modified" amino acid) contains a non-naturally occurring R-group. While "peptide" does not provide a size limitation, smaller length peptides are generally preferred. Smaller length peptides may offer advantages in ease of synthesis and solubility.

Standard one and three letter codes for amino acids are as follows: A=Ala=Alanine: C=Cys=Cysteine: D=Asp=Aspartic acid: E=Glu=Glutamic acid: F=Phe=Phenylalanine: G=Gly=Glycine: H=His=Histidine: I=Ile=Isoleucine: K=Lys=Lysine: L=Leu=Leucine: M=Met=Methionine: N=Asn=Asparagine: P=Pro=Proline: Q=Gln=Glutamine: R=Arg=Arginine: S=Ser=Serine: T=Thr=Threonine: V=Val=Valine: W=Trp=Tryptophan: and Y=Tyr=Tyrosine.

According to an embodiment of the present invention, the peptides or proteins can be modified to contain conservative substitutions. Conservative substitutions for amino acids are shown in Table 1. Factors that can be taken into account for a conservative substitution include amino acid size, charge, polarity, and hydrophobicity. (See, for example, Ausubel, *Current Protocols in Molecular Biology,* John Wiley, 1987-2001, Appendix 1C.) Conservative substitutions, if present, should substantially maintain or enhance either coronavirus inhibition properties or pharmacokinetic properties. In different embodiments, there are 0, 1, 2, 3, 4, 5, or 6-10 conservative substitutions.

Table 1: Conservative Substitutions

Amino Acid	Conservative Substitution
Alanine	D-Alanine, Glycine
Arginine	D-Arginine, Lysine, D-Lysine

Table 1: Conservative Substitutions

Amino Acid	Conservative Substitution
Asparagine	D-Asparagine, Glutamine, D-Glutamine
Aspartic acid	D-Aspartic acid, Glutamic acid, D-Glutamic acid
Cysteine	D-Cysteine
Glutamic acid	D-Glutamic acid, Aspartic acid, D-Aspartic acid
Glutamine	D-Glutamine, Asparagine, D-Asparagine
Histidine	D-Histidine
Isoleucine	D-Isoleucine, Valine, D-Valine, Leucine, D-Leucine
Leucine	D-Leucine, Valine, D-Valine, Isoleucine, D-Isoleucine
Lysine	D-Lysine, Ornithine, D-Ornithine,
Methionine	D-Methionine, Norleucine, D-Norleucine
Phenylalanine	D-Phenylalanine, Tyrosine, D-Tyrosine
Proline	D-Proline
Serine	D-Serine, Threonine, D-Threonine
Threonine	D-Threonine, Serine, D-Serine
Tryptophan	D-Tryptophan, Tryptophan analog*
Tyrosine	D-Tyrosine, Phenylalanine, D-Phenylalanine
Valine	D-Valine, Leucine, D- Leucine, Isoleucine, D-Isoleucine

*The tryptophan analog can be D or L.

According to an embodiment of the present invention, the peptides can be modified, e.g., containing one or more tryptophan analogs and / or one or more lactam bridges.

The present invention also provides retro-inverso analogues of the inhibitors of coronavirus.

As used herein, polypeptide refers to a peptide comprising about 10 to about 500 amino acids. Examples of polypeptides include C-peptides, N-peptides, the chimeric N-peptides, Five Helix, Six Helix, and their derivatives.

According to an embodiment of the present invention, the coronavirus inhibitor is a purified polypeptide. A "purified polypeptide" represents at least 10% of the total polypeptide present in a sample or preparation. In preferred embodiments, the purified polypeptide represents at least about 50%, at least about 75%, or at least about 95% of the total protein in a sample or preparation. Reference to "purified polypeptide" does not require that the polypeptide has undergone any purification and may include, for example, chemically synthesized polypeptide that has not been purified. According to another embodiment of the present invention, the coronavirus inhibitor is a purified oligomer of polypeptide.

One aspect of the present invention describes a method for treating a patient to inhibit coronavirus replication. The method comprises the step of administering to the patient an effective amount of an inhibitor of coronavirus to the patient.

Another aspect of the present invention describes a method of inhibiting the ability of a human coronavirus to infect a cell *in vitro*. The method comprises the step of providing an inhibitor of coronavirus to a cell culture infected with the coronavirus.

Reference to "patient" indicates a mammal undergoing treatment and does not necessarily indicate the presence of a coronavirus infection. Treatment can be provided to a patient infected with the coronavirus to decrease the severity of a coronavirus infection. Treatment can also be provided prophylactically to reduce the likelihood or severity of a coronavirus infection. A patient can be a human or another type of mammal. Treatment of non-human patients may be useful in protecting pets and livestock, or in evaluating the efficacy of a particular treatment.

Reference to "effective amount" indicates an amount sufficient to provide a beneficial effect to a patient. Beneficial effects that can be achieved include detectable inhibition of viral replication or decrease in viral load. The inhibition or decrease should be sufficient to provide a medically significant decrease in the severity or likelihood of a coronavirus infection.

Other features and advantages of the present invention are apparent from the additional descriptions provided herein including the different examples. The provided examples illustrate different components and methodology useful in practicing the present invention. The examples do not limit the claimed invention. Based on the present disclosure the skilled artisan can identify and employ other components and methodology useful for practicing the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the amino acid sequence (SEQ ID NO:1) of the S or spike protein of human coronavirus recently identified in SARS victims, as well as the heptad repeats predicted by two different methods (Multicoil and Learncoil VMF). According to multicoil trimeric prediction (bold), HR1 includes amino acid residues from about 900 to about 974 of SEQ ID NO:1, while HR2 includes amino acid residues from about 1148 to about 1193 of SEQ ID NO:1. According to learncoil VMF prediction (underlined), HR1 includes amino acid residues from about 900 to about 1005 of SEQ ID NO:1, while HR2 includes amino acid residues from about 1151 to about 1185 of SEQ ID NO:1.

Figure 2 shows the amino acid sequences of C34 coil-like inhibitors, also referred to as Heptad Repeat 2 Peptides. The a, d and e residues from the heptad repeat of SARS-HR2 are grafted onto the f, b, and c positions, respectively, of the GCN4 coiled coil to make (Cys)SARS-HR2-GCN4 ((Cys)HR2G, SEQ ID NO:6). Then, through the cysteine residue, this peptide is covalently coupled to (Cys)GCN4

(SEQ ID NO:7) to form a stable dimer exposing the SARS-HR1 binding interface on one side. One example is shown for the SARS coronavirus sequence.

Figure 3 illustrates retro-inverso peptides. The parent linear peptide is shown in the middle (Boxed). The retro-inverso analog is shown in parallel (bottom) and antiparallel (top) orientation. It is apparent that in the antiparallel orientation the side-chain topology is maintained, while the direction of the peptide bond is reversed. In the retro-inverso peptide, all the amino acids are in the (D) configuration.

Figures 4A, 4B, and 4C, provide examples of tryptophan analogs.

DETAILED DESCRIPTION OF THE INVENTION

This invention relates to inhibitors of coronaviruses, such as SARS-CoV. According to an embodiment of the present invention, the inhibitors comprise sequences derived from the heptad repeat regions (e.g., HR1 or HR2) of coronaviruses.

1. The Heptad Repeat Regions of Viral Fusion Proteins

Like other enveloped viruses, coronaviruses have a viral membrane containing glycoproteins that permit the viruses to bind to target cells and achieve membrane fusion, an essential process for viral infection, by which the viral and cellular membranes combine to form a single bilayer. The S glycoprotein on the viral membranes of coronaviruses is such a fusion protein, which participates in receptor binding and membrane fusion. The S protein comprises two heptad repeat regions, HR1 and HR2, which are predicted to collapse to form a trimer of hairpin structure in the fusion process. (See, DeGroot et al. *Journal of Molecular Biology* 196:963-966 (1987); Wolf, E. et al. *Protein Science* 6:1179-1189 (1997); Singh, M. et al. *Journal of Molecular Biology* 290:1031-1041 (1999)).

Heptad repeat regions have been characterized by structural studies in membrane-fusion proteins of a variety of different viruses. In the fusion processes, the heptad-repeat sequences all form trimeric hairpin-like structures, in which a central three-stranded coiled coil is surrounded by supporting helices packed in an antiparallel manner. The trimer-of-hairpins structure is thought to bring the viral and cellular membrane together. The trimer-of-hairpins structure presumably corresponds to the fusion-active state. Extensive studies have shown that viral fusion proteins undergo a series of conformational changes to become the trimer-of-hairpins structure from a pre-hairpin intermediate conformation when exposed to the appropriate triggering signals (Hernandez et al. Annu Rev Cell Dev Biol 12: 627-661 (1996)). Various peptides were designed as exogenous inhibitors to bind to the pre-hairpin intermediate in a dominant-negative manner and consequently block formation of the fusion-active hairpin structures in

viruses such as HIV (Chan and Kim, *Cell* 93: 681-684 (1998); Eckert and Kim, *Annu Rev Biochem* 70: 777-810 (2001)).

The presence of the two heptad-repeat regions, HR1 and HR2, in S2 subunit of coronavirus S proteins indicates that they adopt a trimer-of-hairpins structure in their fusion process. As described herein, analysis predicts that the HR1 and HR2 regions collapse to form the trimer-of-hairpins structure consistent with the structure of HIV gp41. Peptides containing the HR1 and / or HR2 likely act in a dominant-negative manner to prevent the formation of the trimer-of-hairpins structure and inhibit infection of coronaviruses. Agents that prevent conformational changes which play a critical role in viral and cell membrane fusion, such as by stabilizing the native or intermediate states of the viral fusion proteins are expected to prevent fusion activation and block viral entry.

Applicants have analyzed the amino acid sequences of a variety of coronaviruses, including that of the recently identified human coronavirus isolated from SARS victims. (http://ybweb.bcgsc.ca/sars/TOR2_draft_genome_assembly_120403.fasta.gz). SARS-associated coronavirus is also described by Rota *et al.*, Science *300*, 1394-1399 (30 May 2003); published online 1 May 2003, 10.1126/science.1085952; and Marra *et al.*, Science *300*, 1399-1404 (30 May 2003); published online 1 May 2003, 10.1126/science.1085953.

The analysis has shown that coronavirus S protein comprises two heptad repeat regions which are predicted to form a trimer of hairpin structure. As shown in Figure 1, results of analysis of SARS spike protein (SEQ ID NO:1) by two methods (Multicoil and Learncoil VMF) identified two heptad repeat regions that are predicted to form a trimer of hairpin structure as follows.

Predicted by Multicoil, HR1 comprises amino acid residues from about 900 to about 974 of SEQ ID NO:1; and by Learncoil VMF, HR1 comprises amino acid residues from about 900 to about 1005 of SEQ ID NO:1. Predicted by Multicoil, HR2 comprises amino acid residues from about 1148 to about 1193 of SEQ ID NO:1; and by Learncoil VMF, HR2 comprises amino acid residues from about 1151 to about 1185 of SEQ ID NO:1. The composition (identity and order of amino acid residues) of the spike (S) protein of SARS coronavirus presented herein is defined with reference to the TOR2 draft genome assembly provided at the website referenced above.

Based on the understanding of the structures and functions of coronavirus Spike (S) proteins, especially their HR1 and HR2 regions, various coronavirus inhibitors are designed, produced, and tested. Examples of the inhibitors are illustrated as follows.

2. HR2 Peptides

A subject of this invention are peptides, referred to as HR2 (heptad repeat 2) peptides, which comprise at least 20 amino acid residues from coronavirus heptad repeat 2 and are useful as inhibitors of coronavirus infection of mammalian cells, such as human epithelial cells. Such peptides are also referred to as C-peptides, because the S2 subunit is at the C terminus of coronavirus S protein and the HR2 region is C-terminal of the HR1 region (Fig. 1). HR2 peptides are thought to bind to HR1 region in the pre-hairpin intermediate of S protein in a dominant-negative manner, block formation of the fusion-active hairpin structures, and consequently inhibit infection of coronaviruses such as SARS-CoV.

HR2 peptides comprise from about 20 to about 60 amino acid residues from the predicted HR2 region. In some embodiments, HR2 peptides comprise from about 20 to about 50 amino acid residues from the predicted HR2 region. Amino acid residues present in these HR2 peptides may be contiguous amino acid residues that occur sequentially in a coronavirus HR2 or may be noncontiguous amino acid residues that are separated by intervening amino acid residues in the coronavirus HR2. In the embodiments of all peptides described herein in which noncontiguous amino acid residues comprise the peptides, amino acid residues other than those that occur between the noncontiguous residues in the coronavirus (e.g., other than those that occur between the noncontiguous amino acid residues in SARS in nature) are introduced in order to maintain, for example, appropriate distance between the residues and ensure that the resulting peptide has an appropriate conformation. An example of the noncontiguous amino acid residues is shown in Fig. 2 and section 3 as follows.

Specific embodiments of SARS HR2 peptides include any combination of from about 20 amino acid residues to about 60 amino acid residues of SARS HR2 peptide, homologues and variants of such sequences, including variants that result from correction of any errors in the TOR2 draft genome assembly published at http://ybweb.bcgsc.ca/sars/TOR2_draft_genome_assembly_120403.fasta.gz and additional SARS HR2 regions encoded by the genome of additional coronaviruses identified or obtained from SARS victims that differ from the referenced publicly available sequence.

In specific embodiments, SARS HR2 peptides comprise from about 20 to about 60 amino acid residues, which can be contiguous or noncontiguous in the region as it occurs in SARS virus, of the following sequence, which is residues 1144-1200 or 1142-1193 of the predicted spike protein of SARS Tor2 (SEQ ID NO:1).

Amino acid residues 1144-1200 SEQ ID NO:1 has the sequence as follows:
PDVDLGDISGINASVVNIQKEIDRLNEVAKNLNESLIDLQELGKYEQYIKWPWYVWL (SEQ ID NO:2), in which solvent exposed regions of the HR2 include the b, c and f positions of the heptad repeat. The solvent exposed positions are underlined.

Additional peptides can be identified using known methods, such as by determining the location and composition (amino acid sequence) or heptad repeat 2 and selecting peptides of from about 20 to about 60 amino acid residues. Examples of such HR2 peptides are partial or full sequence of SEQ ID NO:2. HR2 peptides can consist of amino acid residues from N to (N+M-1) of SEQ ID NO:2, wherein M is the length of the HR2 peptide, and N can be any integral number from 1 to (58-M).

In specific embodiments, HR2 peptides consist of amino acid residues from 5 to 50, from 9 to 41, from 21 to 57, from 5 to 41, from 30 to 57, or from 1 to 57 of SEQ ID NO:2. The region of residues from 30 to 57 of SEQ ID NO:2 appears to be very highly conserved among coronaviruses and, thus is a peptide of particular interest for designing or identifying C-peptide/HR2 peptide inhibitors.

According to a preferred embodiment of the present invention, the HR2 peptide has a sequence of residues 1148-1182 or 1148-1185 of SEQ ID NO:1. Residues 1148-1182 of SEQ ID NO:1 has the sequence as follows: LGDISGINASVVNIQKEIDRLNEVAKNLNESLIDL (SEQ ID NO:3).

3. HR2 Coil-Like Inhibitors

A further subject of this invention are coronavirus inhibitors, referred to as HR2 coil-like inhibitors, which are stable dimers that expose an HR1 binding site in such a manner that it is available for binding. HR2 coil-like inhibitors are thought to inhibit infection of coronaviruses such as SARS-CoV with the same mechanism as that of HR2 peptides.

In this embodiment, amino acid residues a, d, and e of coronavirus HR2 (e.g., SARS-CoV-HR2) are grafted onto the f, b and c positions, respectively, of a coiled coil, such as the GCN4 coiled coil GCN4-p1, to produce (Cys)-HR2-coiled coil (e.g., (Cys)-SARS-CoV-HR2-GCN4). This peptide is then covalently coupled to (Cys) coiled coil (e.g., (Cys)GCN4) to form a stable dimer exposing the HR1 binding interface of the coronavirus (here, the SARS-CoV-HR1 binding interface) on one side. Examples of such HR2 coil-like inhibitors are shown in Figure 2. GCN4-p1 is a dimeric coiled coil whose sequence was derived from GCN4, a yeast transcription activator (O'Shea, E. K., et al, *Science* 254:539-544 (1991)).

Additional coil-like inhibitors can be identified using similar techniques, which rely on identification of the heptad register, followed by application to the repeat sequence of one or more coil prediction programs (e.g., Multicoil, Learncoil VMF) and grafting of amino acid residues onto an appropriate coiled coil. See, for example, Sia, S. Doctoral Thesis entitled Rational Design of peptide inhibitors of HIV-1 entry. December 2001, submitted to the Committee on Higher Degrees in Biophysics in partial fulfillment of the requirements of Doctor of Philosophy in the subject of Biophysics, Harvard University (see also *Proc Natl Acad Sci USA*;100:9756-61 (2003)).

4. HR1 Peptides

HR1 (Heptad Repeat 1) peptides or inhibitors, also referred to as N-peptide inhibitors, are a further subject of this invention. HR1 peptides are thought to bind to HR2 region in the pre-hairpin intermediate of S protein in a dominant-negative manner, block formation of the fusion-active hairpin structures, and consequently inhibit infection of coronaviruses such as SARS-CoV.

Such HR1 peptides generally include from about 28 to about 60 amino acid residues from coronavirus HR1 peptide, although in some cases, they may include fewer than 28 or more than 60 HR1 amino acid residues. The amino acid residues in the HR1-peptide inhibitors can be residues that occur sequentially (are contiguous) or nonsequentially (are noncontiguous) in the coronavirus HR1 as it occurs in nature. The terms Contiguous and noncontiguous and sequential and nonsequential are used with reference to the amino acid sequence of a peptide or protein (e.g., HR1, HR2) as it occurs in the virus in nature.

A wide variety of HR1 peptides can be identified and produced, using known methods and the present invention encompasses all HR1 peptides from about 28 to about 60 or about 80 amino acid residues from the HR1 repeats described herein. In particular embodiments, HR1 peptides of the present invention include all peptides of from about 28 to about 60 or about 80 amino acid residues of SARS coronavirus HR1, defined with reference to the deduced amino acid sequence encoded by the referenced publicly available SARS genome data (SEQ ID NO:1 and Fig. 1). In addition, the amino acid sequences of HR1 peptides can be altered, such as by addition, substitution or deletion of one or more amino acid residues, in order to alter certain characteristics (e.g., to increase peptide stability, enhance peptide solubility).

HR1 peptides can have a length of from about 28 to about 60 or about 80 amino acid residues, and be derived from residues 900-1005 or 889-972 of SEQ ID NO:1. Examples of HR1 peptides include, but are not limited to: A peptide having a sequence of residues 896-972, 900-938, 914-949, 922-956, 943-980 or 943-981 of SEQ ID NO:1.

Another embodiment of this invention is a complex of an HR1-peptide inhibitor tethered (with an appropriate linker, which can be made of amino acid residues, or can be a chemical or synthetic linker) to a component, such as a ligand (protein or small molecule) that binds coronavirus envelope protein. The complex is useful as a therapeutic agent, because the ligand will increase the effective concentration of the tethered HR1-peptide inhibitor moiety in the vicinity of the coronavirus envelope protein.

HR1 peptides are capable of forming trimeric coiled coil. The HR1 trimeric coiled coil could be stabilized by including cysteine residues to form interchain disulfide bonds in a chemically synthesized or recombinant peptide greater than or equal to 28 and less than or equal to 60 or 80 sequential residues from

the predicted HR1 region. The recombinant versions could contain helix capping residues on the N- and C-terminus. Possible capping residues for the N-terminus are T, D and S. Possible capping residues for the C-terminus are G, R, H, N, or K. Possible amino-terminal caps for the synthetic peptides are acetyl (Ac) or succinimide (suc). For SARS virus, the HR1 comprises the sequence of residues 900-1005. Possible inhibitors of SARS-CoV include, but are not limited to:

CCGTTTSTALGKLQDVVNQNAQALNTLVKQLSSNFGAIS (SEQ ID NO:8),

CCGENQKQIANQFNKAISQIQESLTTTSTALG (SEQ ID NO:9), and

CCGALNTLVKQLSSNFGAISSVLNDILSRLDKVEAEVQIDR (SEQ ID NO:10).

5. HR1 Chimeric Peptides

The present invention also relates to HR1 chimeric peptides comprised of an HR1 peptide fused to a peptide that is capable of forming a solubilizing trimeric coiled coil. The HR1 chimeric peptides form stable and soluble HR1 chimeric trimeric coiled coil. HR1 chimeric peptides are thought to inhibit infection of coronaviruses such as SARS-CoV with the same mechanism as that of HR1 peptides. Examples of the HR1 chimeric peptides include HR1-IQ peptides and HR1-IZ peptides.

5.1. The HR1 peptide portion of the HR1 chimeric peptides

According to an embodiment of the present invention, a HR1 chimeric peptide comprises at least a portion of HR1 of coronavirus S2 protein, as disclosed in sections 1 and 4. The HR1 region component of these peptides can be of varying length and will typically be from about 15 to about 50 amino acid residues from the coronavirus HR1 region. The HR1 peptide, in some cases, may be shorter than 15 amino acid residues or longer than 50 amino acid residues.

5.2. The peptides capable of forming solubilizing trimeric coiled coils

The solubilizing trimeric coiled-coil peptide can be from (comprise amino acid residues that correspond to those in) a variety of sources, including IQ peptides and IZ peptides.

IQ peptide can be from (comprise amino acid residues that correspond to those in) a variety of sources, including yeast transcription activator GCN4, such as GCN4-pII or GCN4-pI$_Q$I; Moloney Murine Leukemia Virus (MoMLV); and the ABC heterotrimer.

The amino acid residues that comprise an IQ peptide of the present invention can be amino acid residues that are sequential (consecutive) or noncontiguous (nonconsecutive) in the trimeric coiled-coil peptide from which it is derived and/or amino acid residues that are sequential (consecutive) or noncontiguous (nonconsecutive) in HR1 peptide, provided that the resulting IQ peptide (the IQ peptide in

which they are present) is stable, soluble, helical, and trimeric under physiological conditions. In the embodiments of IQ peptides in which nonconsecutive amino acid residues of either or both components of the IQ peptide are present, the residues, as included in the IQ peptide, can be consecutive or can be separated or joined by a linker. The linker can be, for example, an amino acid residue(s) that does not occur between two amino acid residues in the peptide from which the component is derived. Alternatively, the "linker" can be a chemical or synthetic linker.

A component of an IQ peptide or HR1-IQ peptide of the present invention is considered to be derived from another peptide (e.g., a trimeric coiled coil or HR1 of a coronavirus) if the component itself (or the nucleic acid molecule(s) that encode the amino acid sequence) is obtained or isolated/separated from a source in which it occurs (e.g., from a cell in which the peptide occurs, such as a portion of a protein from which it can be removed) or is produced by recombinant DNA methods, chemical synthesis or any other method, to comprise an amino acid sequence or a nucleic acid sequence that is the same as or substantially the same as the sequences of the peptide. That is, the term is intended to be interpreted broadly and does not require that a component be physically derived from the peptide referred to.

The trimeric coiled coil peptide component of an IQ peptide must be sufficient in amino acid composition (identity and number/length) to result, when joined to the HR1 peptide portion, in formation of a soluble trimeric helical (coiled-coil) IQ peptide.

In the embodiments in which the soluble IQ peptides comprise an IQ region that is a GCN4 trimeric coiled-coil peptide, they are referred to as IQN peptides. As discussed in section 3, GCN4-p1 is a dimeric coiled coil whose sequence was derived from GCN4, a yeast transcription activator (O'Shea, E. K., et al, *Science* 254:539-544 (1991)). Based on GCN4-p1, trimeric coiled-coil peptides have been developed, such as GCN4-pII (Harbury, P. B., et al, *Nature* 371:80–3 (1994)) and GCN4-pI_QI (Eckert D.M., et al, *J. Mol. Biol.* 284:859–65 (1998)). The trimeric coiled-coil peptides can be used to fuse with the HR1 sequence.

The chimeric HR1-IQN peptides comprise all or a portion of GCN4-pI_Q I or a modified version of all or a portion of GCN4-pI_QI, such as a modified portion that includes mutations for increased solubility, and an HR1 peptide of from about 15 to about 50 amino acid residues.

In certain embodiments of the IQN peptides of the present invention, the trimeric coiled-coil peptide, referred to as the "GCN4 portion", comprises at least 15, 16, 17, 18, 19 or 20 amino acid residues of GCN4 (S. K. Sia and P. S. Kim, *Proc. Natl. Acad. Sci. U.S.A.* 100:9756-9761 (2003)). The amino acid residues present in the components of an IQN peptide can correspond to amino acid residues that are sequential (consecutive) or nonsequential (nonconsecutive) in, respectively, the GCN4 transcription activator (or GCN4-pI_QI) and HR1 peptide or a modified version of the activator or the HR1 peptide, provided that the resulting IQN peptide is an inhibitor of coronavirus infection of cells, as described

herein. The IQ and IZ peptides of the present invention can be produced as a continuous peptide or as components that are joined or linked after they are formed. As used herein, the terms "joined" or "joined in such a manner" or "incorporated" include incorporating amino acid residues by either approach.

For example, the GCN component of an IQN peptide can comprise consecutive amino acid residues from GCN4-pI$_Q$I, modified, if desired (e.g., to increase solubility). Alternatively, amino acid residues that are not consecutive in the GCN4 activator (or in GCN4-pI$_Q$I), joined in such a manner that they are nonconsecutive or consecutive in the resulting GCN4 component of an IQN peptide, can be incorporated in the IQN peptide. Similarly, the amino acid residues of the HR1 peptide component of an IQN peptide of the present invention can be amino acid residues that occur consecutively or nonconsecutively in HR1 peptide of SARS S protein and can be incorporated into in IQN peptide in such a manner that they are consecutive or nonconsecutive in the resulting peptide. In the embodiments in which nonconsecutive amino acid residues are used, they can be separated by one or more "linker" molecules, if needed to retain the respective functions/characteristics of the components and of the IQN peptide. For example, an amino acid residue(s) other than the residue(s) that normally occur between two amino acid residues of GCN4 or HR1 peptide can be used to link or join the two amino acid residues in the IQN peptide. Alternatively, the linker can be a chemical or synthetic linker, for example. IQN36, as well as versions of IQN17 that are shortened in the 'IQ' region, are also described. These shortened versions may be therapeutically advantageous because, for example, they are easier and less expensive to produce than are larger peptides.

The examples of IQ peptides include:

GCN4-pI$_Q$I: RMKQIEDKIEEILSKQYHIENEIARIKKLIGER (SEQ ID NO:11)

GCN4-pI$_Q$I': RMKQIEDKIEEIESKQKKIENEIARIKKLIGERY (SEQ ID NO:12)

A specific embodiment of an IQN peptide is IQN17, which contains 29 residues of GCN4-pI$_Q$I, including three mutations for increased solubility. The sequence of IQN17 consists of residues 1-29 of SEQ ID NO:12.

Shortened versions of IQN17, which each include a shorter GCN component than is present in IQN17, are also the subject of this invention. Specific examples of these shortened IQN17 peptides are:

a) shortened IQN17 #1, consisting of residues 22-29 of SEQ ID NO:12;

b) shortened IQN17 #2, consisting of residues 15-29 of SEQ ID NO:12;

c) shortened IQN17 #4, consisting of residues 8-29 of SEQ ID NO:12;

d) shortened IQN17 #5, consisting of residues 8-28 of SEQ ID NO:12; and

e) shortened IQN17 #3, in which there are 15 non-HR1 amino acid residues KIKKIENEIARIKKL (SEQ ID NO:13). This is GCN4-pI$_Q$I' with a Q to I mutation, and is referred to as GCN4-pII'.

The solubilizing trimeric coiled-coil peptide can also be from other designed trimeric coiled coils, such as the isoleucine zipper (IZ) described by Tanaka et al. (Suzuki, K., Hiroaki, H., Kohda, D. & Tanaka, T. *Protein Eng.* 11:1051–1055 (1998)), or derivatives of this 'IZ' sequence. In those embodiments in which the trimeric coiled-coil peptide is from the isoleucine zipper, they are referred to as IZ peptides.

Tanaka's isoleucine zipper: Ac-YGGIEKKIEAIEKKIEAIEKKIEAIEKKIEA-NH_2 (SEQ ID NO:14)

The "IZ" molecule derivative: Ac-YGGIKKEIEAIKKEQEAIKKKIEAIEKEIEA-NH_2 (SEQ ID NO:15).

Moreover, the solubilizing trimeric coiled-coil peptide can comprise a trimeric coiled-coil peptide from HIV.

5.3. HR1 chimeric peptide configurations

In one embodiment, the components of the HR1-IQ peptides are present in the following "order": N-terminus--trimeric coiled-coil peptide--HR1 peptide-- C-terminus. Alternatively, the order of components can be reversed: N-terminus-HR1 peptide-trimeric coiled-coil peptide-C-terminus.

HR1-IQN17 and other HR1-IQN peptides can be tested for their ability to inhibit coronavirus infection of cells using known methods.

Soluble HR1 chimeric peptides of the present invention comprise, in specific embodiments, the amino acid sequences shown as follows:

TIKKEIEAIKKEQEAIKKKIEAIEKEITTTSTALGKLQDVVNQNAQALNTLVKQLSSNFGAISG (SEQ ID NO:16), or a peptide consisting of residues 2-63 of SEQ ID NO:16;

TRMKQIEDKIEEIESKQKKIENEIARIKKLITTTSTALGKLQDVVNQNAQALNTLVKQLSSNFGAISG (SEQ ID NO:17), or a peptide consisting of residues 2-67 of SEQ ID NO:17;

RMKQIEDKIEEIESKQKKIENEIARIKKLISQIQESLTTTSTALGKLQDVVNQNAQALNTLVKQLS (SEQ ID NO:18);

RMKQIEDKIEEIESKQKKIENEIARIKKLIENQKQIANQFNKAISQIQESLTTTSTALG (SEQ ID NO:19);

TRMKQIEDKIEEIESKQKKIENEIARIKKLENQKQIANQFNKAISQIQESLTTTSTALG (SEQ ID NO:20);

TIKKEIEAIKKEQEAIKKKIEAIEKEIENQKQIANQFNKAISQIQESLTTTSTALG (SEQ ID NO:21), or a peptide consisting of residues 2-56 of SEQ ID NO:21;

TIKKEIEAIKKEQEAIKKKIEAIEKRLQSLQTYVTQQLIRAAEIRASAN (SEQ ID NO:22), or a peptide consisting of residues 2-49 of SEQ ID NO:22;

TRMKQIEDKIEEIESKQKKIENEIARIKKRLQSLQTYVTQQLIRAAEIRASAN (SEQ ID NO:23), or a peptide consisting of residues 2-53 of SEQ ID NO:23;

TRMKQIEDKIEEIESKQKKIENEIARIKKALNTLVKQLSSNFGAISSVLNDILSRLDKVEAEVQIDR (SEQ ID NO:24), or a peptide consisting of residues 2-67 of SEQ ID NO:24;

TIKKEIEAIKKEQEAIKKKIEAIEKALNTLVKQLSSNFGAISSVLNDILSRLDKVEAEVQIDR (SEQ ID NO:25), or a peptide consisting of residues 2-63 of SEQ ID NO:25.

IKKEIEAIKKEQEAIKKKIEAIEKEISQIQESLTTTSTALGKLQDVVNQNAQALNTLVKQLS (SEQ ID NO:26); and

IKKEIEAIKKEQEAIKKKIEAINVLYENQKQIANQFNKAISQIQESLTTTSTALGKLQ (SEQ ID NO:27).

Examples of IZ peptides, referred to as IZN36, IZN23 and IZN17-like inhibitors of SARS coronavirus are shown as follows.

IKKEIEAIKKEQEAIKKKIEAIEKEIENQKQIANQFNKAISQIQESLTTTSTALGKLQDVVN (SEQ ID NO:28), or a peptide consisting of residues 1-49 of SEQ ID NO:28;

IKKEIEAIKKEQEAIKKKIEAIEKEINQNAQALNTLVKQLSSNFGAISSVLNDILSRLDKVE (SEQ ID NO:29), or a peptide consisting of residues 1-49 of SEQ ID NO:29;

IKKEIEAIKKEQEAIKKKIEAIEKSLTTTSTALGKLQDVVN (SEQ ID NO:30);

IKKEIEAIKKEQEAIKKKIEAIEKAISSVLNDILSRLDKVE (SEQ ID NO:31);

SQIQESLTTTSTALGKLQDVVNQNAQALNTLVKQLSSIKKEIEAIKKEQEAIKKKIEAIEKEIG (SEQ ID NO:32)

Such inhibitor peptides can be administered to an individual, such as in a pharmaceutical composition comprising one or more (at least one) of the IZ peptides and an appropriate carrier (e.g., an appropriate buffer or carrier) to prevent or inhibit SARS infection in the individual.

6. Five-Helix Proteins

Another embodiment of the present invention encompasses Five-Helix or Five-Helix proteins. The Five-Helix proteins comprise three heptad repeat 1 (HR1) components and at least two and up to, but not including, three heptad repeat 2 (HR2) components of coronavirus S2 protein, and are soluble under physiological conditions. The Five-Helix proteins interact with or bind to HR2 of coronavirus S2 protein; and inhibit coronavirus infection of mammalian cells, particularly human cells, such as epithelial cells of the upper respiratory tract or gastrointestinal tract. Thus, Five-Helix includes three HR1 components and at least two HR2 components of coronavirus S2 protein. It can also include a portion of a third

coronavirus S2 HR2, but does not comprise three entire S2 protein HR2 components. In each case, the HR1 and HR2 components are separated by linkers, preferably amino acid residue linkers, which can be of any length. The amino acid linkers can be, for example, GGGSSGGGSGG (SEQ ID NO:33).

Also the subject of the present invention is Six-Helix protein, which comprises three HR1 components and three HR2 components of coronavirus S protein, joined by linkers, such as amino acid residue linkers. In other embodiments, the amino acid sequence of Six-Helix differs from those shown in the figure by addition, deletion, substitution or alteration of at least one amino acid residue. Six-Helix protein is useful not only for producing Five-Helix, but also as a negative control in screening for drugs that inhibit coronavirus infection of cells.

In one embodiment, a Five-Helix protein of this invention comprises three heptad repeat 1 (HR1) components and at least two and up to, but not including, three heptad repeat 2 (HR2) components of an S2 protein of a coronavirus that causes SARS; interacts with or binds to HR2 of the SARS-CoV S2 protein; and inhibits infection of human cells, such as infection of cells of the epithelium of the respiratory tract.

In one embodiment, Five-Helix can be represented as: HR1-linker-HR2-linker-HR1-linker-HR2-linker-HR1, wherein HR1 represents a coronavirus HR1 and HR2 represents a coronavirus HR2. In a further embodiment, Five-Helix can be represented as: HR1-linker-HR2-linker-HR1-linker-HR2-linker-HR1-linker-partial HR2 wherein HR1 represents a coronavirus HR1 and HR2 represents a coronavirus HR2 and "partial HR2" indicates that this component is a portion of a coronavirus HR2. The term Five-Helix or Five-Helix protein encompasses all such embodiments (those including three HR1 components and two or more, but less than three, HR2 components, separated by appropriate linkers). In specific embodiments, the HR1 and HR2 components are human coronavirus heptad repeats and partial HR2 is a partial (less than a complete) human coronavirus heptad repeat.

In further specific embodiments, the Five Helix protein can be represented as: HR1 of SARS virus-linker-HR2 of SARS virus-linker-HR1 of SARS virus-linker-HR2 of SARS virus-linker-HR1 of SARS. In further specific embodiments, the Five Helix protein can be represented as: HR1 of SARS virus-linker-HR2 of SARS virus-linker-HR1 of SARS virus-linker-HR2 of SARS virus-linker-HR1 of SARS-linker-partial HR2 of SARS.

In specific embodiments, the Five-Helix proteins comprise HR1 components having the sequence of from about residue 900 to about residue 1013 of SEQ ID NO:1, HR2 components having the sequence of residues from about 1148 to about 1193 of SEQ ID NO:1, and amino acid linkers therebetween. The HR1 and/or HR2 SARS sequence can be altered by the addition, deletion or substitution of one or more amino acid residues to produce additional Five-Helix proteins.

In a specific embodiment, a Five-Helix protein comprises three HR1 repeats having the following amino acid sequence: the residues 900-1005 of SEQ ID NO:1, and at least two and up to, but not including, three HR2 repeats having the following amino acid sequence: the residues 1151-1185 of SEQ ID NO:1. In another embodiment, a Five-Helix protein comprises three HR1 repeats having the following amino acid sequence: the residues 900-974 of SEQ ID NO:1, and at least two and up to, but not including, three HR2 repeats having the following sequence: the residues 1148-1193 of SEQ ID NO:1. In another specific embodiment, a Five-Helix protein comprises three HR1 repeats having the amino acid sequence: the residues 900-1005 of SEQ ID NO:1, and at least two and up to, but not including, three HR2 repeats having the following sequence: the residues 1148-1193 of SEQ ID NO:1. In a further specific embodiment, a five-helix protein comprises three HR1 repeats having the amino acid sequence: the residues 900-974 of SEQ ID NO:1, and at least two and up to, but not including, three HR2 repeats having the following amino acid sequence: the residues 1151-1185 of SEQ ID NO:1.

According to an embodiment of the present invention, a Five-Helix protein has the sequence of residues 1-243 of SEQ ID NO:34, whereas the corresponding Six-Helix the sequence of SEQ ID NO:34:

MVLYENQKQIANQFNKAISQIQESLTTTSTALGKLQDVVNQNAGGGSSGGGSGG**DISGINASVVNIQKEI DRLNEVAKNLNESLIDLQEL**GGGSSGGGSGGVLYENQKQIANQFNKAISQIQESLTTTSTALGKLQDVVN QNAGGGSSGGGSGG**DISGINASVVNIQKEIDRLNEVAKNLNESLIDLQEL**GGGSSGGGSGGVLYENQKQI ANQFNKAISQIQESLTTTSTALGKLQDVVNQNAGGGSSGGGSGG**DISGINASVVNIQKEIDRLNEVAKNL NESLIDLQEL** (SEQ ID NO:34).

According to another embodiment of the present invention, a Five-Helix protein has the sequence of residues 1-237 of SEQ ID NO:35, whereas the corresponding Six-Helix the sequence of SEQ ID NO:35:

MTALGKLQDVVNQNAQALNTLVKQLSSNFGAISSVLNDILSGGGSSGGGSGG**DISGINASVVNIQKEIDR LNEVAKNLNESLIDLQEL**GGGSSGGGSGGTALGKLQDVVNQNAQALNTLVKQLSSNFGAISSVLNDILSG GGSSGGGSGG**DISGINASVVNIQKEIDRLNEVAKNLNESLIDLQEL**GGGSSGGGSGGTALGKLQDVVNQN AQALNTLVKQLSSNFGAISSVLNDILSGGGSSGGGSGG**DISGINASVVNIQKEIDRLNEVAKNLNESLID LQEL** (SEQ ID NO:35).

According to an alternative embodiment of the present invention, a Five-Helix protein has the sequence of residues 1-237 of SEQ ID NO:36, whereas the corresponding Six-Helix the sequence of SEQ ID NO:36:

MQIDRLITGRLQSLQTYVTQQLIRAAEIRASANLAATKMSEGGGSSGGGSGG**DISGINASVVNIQKEIDR LNEVAKNLNESLIDLQEL**GGGSSGGGSGGQIDRLITGRLQSLQTYVTQQLIRAAEIRASANLAATKMSEG GGSSGGGSGG**DISGINASVVNIQKEIDRLNEVAKNLNESLIDLQEL**GGGSSGGGSGGQIDRLITGRLQSL

QTYVTQQLIRAAEIRASANLAATKMSEGGGSSGGGSGG**DISGINASVVNIQKEIDRLNEVAKNLNESLID LQEL** (SEQ ID NO:36).

According to an alternative embodiment of the present invention, a Five-Helix protein has the sequence of residues 1-317 of SEQ ID NO:37, whereas the corresponding Six-Helix the sequence of SEQ ID NO:37:

MGIGVTQNVLYENQKQIANQFNKAISQIQESLTTTSTALGKLQDVVNQNAQALNTLVKQLSGGGSSGGGS GG**LGDISGINASVVNIQKEIDRLNEVAKNLNESLIDLQELGKYEQYIK**GGGSSGGGSGGGIGVTQNVLYE NQKQIANQFNKAISQIQESLTTTSTALGKLQDVVNQNAQALNTLVKQLSGGGSSGGGSGG**LGDISGINAS VVNIQKEIDRLNEVAKNLNESLIDLQELGKYEQYIK**GGGSSGGGSGGGIGVTQNVLYENQKQIANQFNKA ISQIQESLTTTSTALGKLQDVVNQNAQALNTLVKQLSGGGSSGGGSGG**LGDISGINASVVNIQKEIDRLN EVAKNLNESLIDLQELGKYEQYIK** (SEQ ID NO:37).

The amino acid sequence of each of the components (the HR1 repeats and the HR2 repeats) can vary widely, provided that the resulting repeats form a trimer of hairpins. The sequences provided herein can be modified by the addition, deletion, or substitution of one or more amino acid(s). Amino acid residues in the C-peptide helix (HR2), particularly the amino acid residues that are solvent exposed (have high solvent accessibility) in the trimer of hairpin structure, can be changed to other amino acid residues with considerable flexibility. For example, such amino acid residues can generally be changed to (substituted with) naturally-occurring amino acid residue except, in general, proline, which can be used as a cap. Amino acid residues in the N-peptide helix (HR1) can also be changed. In the N-peptide, the amino acid residues that are buried in the coiled-coil trimer (buried in the trimer interface) can be substituted with amino acid residues that can be accommodated in the interface. Such characteristics of amino acid as size, charge and hydrophobicity must be taken into consideration in selecting amino acid substitutions in the N-peptide.

As discussed above, the amino acid residue composition of Five-Helix of the present invention can vary greatly. In those instances in which Five-Helix is used in therapeutic applications, it must present a surface or region that is available for binding or interacting with coronavirus HR2. That is, the remaining surface of Five-Helix must be present in such a manner or conformation that it is available to bind or interact with coronavirus HR2. In the cases in which Five-Helix is used as a drug-screening tool or an antibody-screening tool or as a vaccine or immunogen, Five-Helix need not bind or interact with coronavirus HR2.

The HR1 components of Five-Helix proteins of the present invention can vary in amino acid composition (type and number). They will generally comprise from about 20 to about 100 amino acid residues of HR1 of coronavirus (e.g., human) S2 protein and each component can comprise any number of amino acid residues from about 20 to about 100. They will generally comprise from about 20 to about

60 amino acid residues of HR2 of coronavirus (e.g., human) S2 protein and each can comprise any number of amino acid residues from about 20 to about 60. The amino acid composition of each HR1 component in a Five-Helix protein can be the same or different. The amino acid composition of each HR2 component in a Five-Helix protein can be the same or different. The amino acid residues included in HR1 and HR2 can occur sequentially/consecutively in the corresponding region of a coronavirus or may be nonconsecutive amino acids (e.g., amino acids that are not consecutive in the coronavirus HR1 and/or HR2 can make up an HR1 or HR2 of a Five-Helix).

In further embodiments, Five-Helix protein can comprise equivalent HR1 and HR2 components from other coronaviruses, which can be identified using known methods, such as Multicoil and Learncoil VMF. See, for example, Wolf, E. et al. Protein Science (June 1997) 6: 1179-1189, describing Multicoil and Singh, M. et al. Journal of Molecular Biology (1999) 290: 1031-1041, describing Learncoil VMF. Alternatively, Five-Helix proteins of the present invention can differ from a sequence presented herein by addition, deletion, substitution or alteration of at least one amino acid residue. The linkers can be of any length or composition, provided that the Five-Helix protein conformation, described herein, is retained. Five-Helix can be an L-amino acid protein, a D-amino acid protein or a combination of L-amino acid residues and D-amino acid residues; these residues can be modified residues or non naturally occurring residues.

7. The Retro-Inverso Analogues

The present invention further relates to the inhibitors that are retro-inverso analogues of the inhibitors derived from HR1 and/or HR2 of the coronaviruses such as SARS-CoV. The *in vivo* stability of a peptide is critical for its application in therapies. The retro-inverso analogues have higher stability than their counterparts, while reserving the capability of binding the pre-hairpin intermediate of S protein and inhibiting coronavirus inhibition.

7.1. The Retro-Inverso Peptides

The retro-inverso analogue of a peptide is more stable than the peptide, while it often maintains the biological activities of the peptide. A retro-inverso peptide is an isomer of a linear peptide in which the direction of the amino acid sequence is reversed, and the chirality of each amino acid residue is inverted (Goodman & Chorev, *Acc. Chem. Res.* 12:1-7 (1979); Chorev & Goodman, M. *Acc. Chem. Res.* 26:266-273 (1993); Chorev & Goodman, M. *Trends Biotechnol.* 13:438-445 (1995); Fletcher & Campbell, *Chem. Rev.* 98:763-795(1998); Taylor, et al., *J. Pharmacol. Exp. Ther.* 295:190-194 (2000)). This results in inversion of each peptide bond within the peptide sequence, while the overall topology of

the side-chains is maintained, except for the terminal groups. Hence, when a retro-inverso peptide is superimposed onto the parent peptide in an antiparallel fashion, the overall topology of the side-chains is maintained (See Fig. 3).

Because most proteins are composed of L-amino acids, retro-inverso peptides are usually peptides composed of D-amino acids. As used herein, D-chirality is indicated with the lowercase, while L-chirality is indicated with the uppercase. For amino acids comprising more than one chiral center, like isoleucine and threonine, the amino acid used in the retro-inverso analog may comprise any combination of the possible configurations of the said amino acid. As used herein, "i" may represent (D)-Isoleucine, (D)-allo-isoleucine, or (L)-allo-isoleucine, while "t" may represent (D) threonine, (D)-allo-threonine, or (L)-allo-threonine.

When the interaction of a peptide ligand with another peptide or protein is mainly dependent on side-chain topology, and much less so on contacts established with the peptide amide backbone, the binding affinity of the parent sequence can be preserved in the retro-inverso analog. There are several examples of retro-inverso peptides, which maintain the same biological activity of the parent peptides (for recent examples see Levi *et al.*, *Res. Human Retroviruses* 16:59-65 (2000); Tayor *et al.*, 2000; Pescarolo *et al.*, *FASEB J.* 15:31-33 (2000); Chen *et al.*, *J. Med. Chem.* 45 1624-1632 (2002); D'Ursi *et al.*, *J. Med. Chem.* 46 1807-1810 (2003)). In contrast, retro-inverso analogs were biologically inactive in a few cases. A possible cause might be the non-complementarity of the end group with the corresponding end groups of the linear peptide, as for (Fletcher & Campbell, *Chem. Rev.* 98:763-795(1998)).

This non-complementarity, however, is unlikely to render inactive the retro-inverso analogues of the heptad repeats of viral fusion proteins. These peptides are thought to act in a dominant-negative manner, by substituting for one or more components of the trimer, which results from collapse of the HR 1 and 2, thereby preventing the conformational changes necessary for fusion (Lambert et al., *Proc. Natl. Acad. Sci. U.S.A.* 93:2186-2191 (1996); Chan & Kim, *Cell* 93:681-684 (1998); Sodroski, *Cell* 99:243-246 (1999)). This interaction occurs over a large surface area, and does not depend on a single, specific contact between the interacting peptide pairs. For instance, N-terminal truncations of the potent HIV-1 fusion inhibitor DP-178 give rise to fully active peptides (Lawless *et al.*, *Biochemistry* 35:13697-13708 (1996)).

Furthermore, retro-inverso peptides invariably show extended stability in serum, because the inverted amide backbone makes them poor substrates for serum proteases (Goodman & Chorev, *Acc. Chem. Res.* 12:1-7 (1979); Chorev & Goodman, M. *Acc. Chem. Res.* 26:266-273 (1993); Chorev & Goodman, M. *Trends Biotechnol.* 13:438-445 (1995); Levi *et al.*, *Res. Human Retroviruses* 16:59-65 (2000); Tayor *et al.*, 2000; Pescarolo *et al.*, *FASEB J.* 15:31-33 (2000); Chen *et al.*, *J. Med. Chem.* 45 1624-1632 (2002); D'Ursi *et al.*, *J. Med. Chem.* 46 1807-1810 (2003)). The increased stability in serum

is a key advantage of retro-inverso peptides, since it may result in increased serum half-life and improved PK properties. Indeed, D'Ursi *et al. J. Med. Chem.* 46 1807-1810 (2003)) show that the retro-inverso analog of an octapeptide inhibitor of Feline Immunodeficiency Virus is only 4-fold less active than the parent peptide in vitro, but it is *6-fold more potent* in the presence of 50% Normal Cat Serum.

7.2. The Retro-Inverso Analogues of Coronavirus HR Peptides

According to an embodiment of the present invention, the inhibitor comprises a retro-inverso analogue of any of the inhibitory peptides or proteins disclosed herein, including C-peptides, N-peptides, Five-Helix proteins, Heptad Repeat 2 (HR2) peptides, and Heptad Repeat 1 (HR1) coiled-coil peptides. The inhibitor comprises a retro-inverso analogue of a sequence from coronavirus heptad repeat 1 or heptad repeat 2.

According to an embodiment of the present invention, the coronavirus is a human coronavirus or a mouse coronavirus. According to a preferred embodiment of the present invention, the coronavirus is a human SARS coronavirus (SARS-CoV).

According to an embodiment of the present invention, the inhibitor comprises a retro-inverso analogue of a sequence from coronavirus heptad repeat 2 (HR2); the sequence has a length of at least 20 amino acid residues. According to a preferred embodiment of the present invention, the inhibitor is a retro-inverso analogue of HR2 peptide or C-peptide disclosed herein. The inhibitor comprises a retro-inverso analogue of the predicted HR2 region. The HR2 sequence has a length of from about 20 to about 60 amino acid residues, or preferably from about 20 to about 50 amino acid residues. Amino acid residues present in these inhibitors may be a retro-inverso sequence of amino acid residues that occur sequentially in a coronavirus HR2 or may be amino acid residues that are separated by other (intervening) amino acid residues in the coronavirus HR2.

Like corresponding HR2 inhibitors, the inhibitors can bind to the HR1 regions of the pre-hairpin intermediate in a dominant-negative manner to block formation of the hairpin structures, and thereby prevent the infection of coronavirus to the cells.

Possible inhibitors include, but are not limited to, peptides comprising a retro-inverso analogue of the amino acid sequences disclosed in sections 2-5 and peptides that have an amino acid sequence sufficiently similar to any of those sequences to function in the same manner. In particular embodiments, a inhibitor comprises a retro-inverso analogue of some or all of the amino acid sequence of SARS virus HR2, as disclosed in section 2. The SARS fusion inhibitors comprise from about 20 amino acid residues to about 60 amino acid residues. Amino acid residues present in these retro-inverso inhibitors may be a sequence of amino acid residues that occur consecutively (contiguously) in a coronavirus HR2 or may be

amino acid residues that are separated by other (intervening) amino acid residues noncontiguously) in the coronavirus HR2.

The inhibitor can comprise a retro-inverso analogue of any of the sequences derived from SEQ ID NO:2, as disclosed in section 2. According to an embodiment of the present invention, the inhibitor can be any of the sequences derived from the retro-inverso analogue of SEQ ID NO:2, which is lwvywpwkiyqeykgleqldilsenlnkavenlrdiekqinvvsanigsidgldvdp. Examples of such HR2 retro-inverso analogues are partial or full sequence of the retro-inverso analogue of SEQ ID NO:2. HR2 retro-inverso analogues can consist of amino acid residues from N to (N+M-1) of the analogue, wherein M is the length of the HR2 peptide, and N can be any integral number from 1 to (58-M).

According to a further preferred embodiment of the present invention, the inhibitor comprises ldilsenlnkavenlrdiekqinvvsanigsidgl, which is the retro-inverso analogue of SEQ ID NO:3.

According to an embodiment of the present invention, the fusion inhibitor of a coronavirus comprises a retro-inverso analogue of a sequence of at least 28 amino acid residues from coronavirus heptad repeat 1 (HR1). Like corresponding HR2 inhibitors, the retro-inverso inhibitors can bind to the HR1 regions of the pre-hairpin intermediate in a dominant-negative manner to block formation of the hairpin structures, and thereby prevent the infection of coronavirus to the cells. The inhibitor is retro-inverso HR1 peptide or N-peptide. The inhibitor can be the retro-inverso analogue of any of the HR1 or N-peptide inhibitors disclosed herein.

According to an alternative embodiment of the present invention, the inhibitor comprises a retro-inverso analogue of soluble HR1-IQ peptides or HR1-IZ peptides, which are comprised of a soluble trimeric coiled coil fused to an HR1 peptide, which has the sequence of at least a portion of HR1 of coronavirus S2 protein. The peptides comprise a peptide of at least 15 amino acid residues from HR1 of a coronavirus fused in the proper heptad repeat register to a soluble trimeric coiled coil, such as GCN4-pIqI or IZ. The HR1 region component of these peptides can be of varying length and will typically be from about 15 to about 50 amino acid residues from the coronavirus HR1 region.

The inhibitor can be the retro-inverso analogue of any of the HR1 coiled-coil inhibitors disclosed herein.

According to a further preferred embodiment of the present invention, the inhibitor comprises a peptide having a retro-inverso analogue of sequence selected from the group consisting of the partial or full sequence of SEQ ID NO: 16-28 .

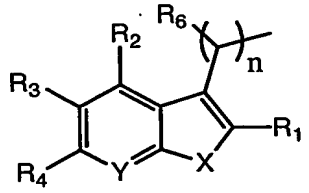
8. Inhibitors Containing Tryptophan Analogs

The present invention further relates to C-peptide and N-peptide coronavirus inhibitors containing one or more tryptophan analogs.

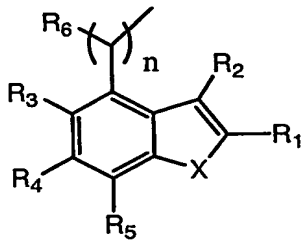
A variety of different C-peptide and N-peptide inhibitors can be obtained based on the disclosure provided herein including the identification of HR1 and HR2 sequences, and the use of tryptophan analogs. Tryptophan analogs can be used to increase serum half-life and improve pharmacokinetic properties. Additional regions that may be present include additional coronavirus regions and other types of regions such as enhancer regions. Enhancer regions either substantially maintain or enhance either coronavirus inhibitor properties or pharmacokinetic properties. Examples of enhancer regions are provided in Barney *et al.*, International Publication Number WO 01/03723, published January 18, 2001.

8.1. Tryptophan Analogs

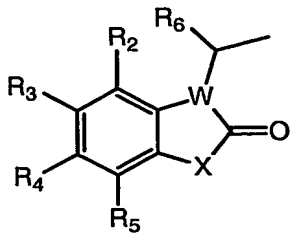
Tryptophan analogs can be present in either D or L stereochemistry. Preferred tryptophan analogs are modified amino acids having an R group (D or L stereochemistry) independently selected from the group consisting of:



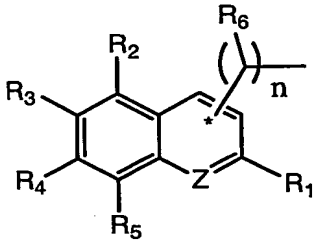
I



II



III



IV

wherein X is O, S, or NR_7;

Y is C-R_5 or N;

W is N or CH;

Z is CH or N;

R_1, R_2, and R_5 are independently selected from the group consisting of hydrogen, halogen, C_1-C_6 alkyl, C_1-C_6 alkylamino, amino, and carboxyl;

R_3 and R_4 are either (1) independently selected from the group consisting of hydrogen, halogen, C_1-C_6 alkyl, C_1-C_6 alkylamino, amino, and carboxyl; or (2) joined together to provide a fused benzene ring;

R_6 is H or methyl;

R_7 is H or linear, branched or cyclic C_1-C_6 alkyl; and

n is 0 or 1;

further provided that in the case of formula I, R_1 may also be CH_2 where there is a bond from this CH_2 to the alpha-NH of the modified amino acid.

Specific examples of different tryptophan analogs are shown in Figures 4A, 4B and 4C. The tryptophan analogs are shown as amino acids. Amino acids are joined together in a peptide by peptide bonds.

8.2. Additional HR Peptide Structures

Preferably, enhancer regions are shorter length amino acid sequences up to about eight amino acids. In an embodiment of the present invention, the enhancer region at the N-terminus is XQEXEQK (SEQ ID NO:38), whereas the enhancer region at the C-terminus is XPXYVXL (SEQ ID NO:39), where each X is independently either tryptophan or a tryptophan analog.

According to an embodiment of the present invention, tryptophan analogs may be present in the HR2 based region or an additional amino acid region located at the N-terminus or C-terminus of the HR2 based region.

In an embodiment of the present invention the C-peptide consists of:

(a) a HR2 based region comprising about 18 amino acids of SEQ ID NO: 2 optionally modified with one or more conservative substitutions thereof;

(b) an optionally present N-terminus enhancer group joined to the N-terminus of the HR2 based region;

(c) an optionally present C-terminus enhancer group joined to the C-terminus of the HR2 based region;

provided that at least one tryptophan analog is present,

further provided that the C-peptide N-terminus, C-terminus, or both the N-terminus and C-terminus may contain a protecting group.

In a more preferred embodiment a C-peptide consists of

(a) a HR2 based region consisting of a sequence selected from the group consisting of:

amino acid residues from N to (N+M-1) of SEQ ID NO:2, wherein M is the length of the HR2 peptide, and N can be any integral number from 1 to (58-M).

(b) an optionally present N-terminus enhancer group, preferably WQEWEQKI (SEQ ID NO:40), where one or both tryptophans may be replaced with a tryptophan analog;

(c) an optionally present C-terminus enhancer group, preferably, WPWYVWL (SEQ ID NO:41) where one, two or all three tryptophans may be replaced with a tryptophan analog;

provided that at least one tryptophan analog is present; and

provided that the C-peptide N-terminus, C-terminus, or both the N-terminus and C-terminus may contain a protecting group.

In a further preferred embodiment, According to a preferred embodiment of the present invention, the HR2 peptide has a sequence of Z-LGDISGINASVVNIQKEIDRLNEVAKNLNESLIDLXPXYVXL (SEQ ID NO:42), and

a variant thereof comprising one or more conservative substitutions;

wherein Z is an optionally present enhancer group, wherein each X is independently either tryptophan or a tryptophan analog;

provided that the C-peptide N-terminus, C-terminus, or both the N-terminus and C-terminus may contain a protecting group.

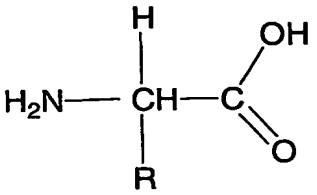
9. Inhibitors Containing Lactam Bridges

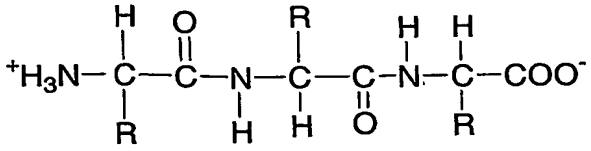
The introduction of one or more lactam bridges into a C-peptide or N-peptide can be used to stabilize a conformation providing advantages over the corresponding structure not containing lactam bridges. Advantages that can be provided include one or more of the following; resistance to proteolytic enzyme, improved serum half-life, enhanced biological activity, and enhanced pharmacokinetic properties. The same enhanced stability, coupled with cross-reactivity of the antibody response between the linear and the corresponding cyclic constrained sequences, may make the peptides more suitable than their linear counterparts for generation of an immune response as part of a vaccine.

9.1. Lactam Bridges

A lactam bridge is formed between a carboxyl moiety of a first amino acid and an amino moiety of a second amino acid. The carboxyl and amino moieties form an amide bond.

The general structure of an individual amino acid and amino acids in a peptide (tripeptide with ionized amino and carboxyl groups) are as follows (where R is an amino acid R group):





Introduction of lactam bridges into a particular peptide can make use of amino acids having suitable groups or involve modifying the peptide to insert one or more suitable amino acids. Suitable amino acids for forming a lactam bridge include naturally occurring amino acid and non-naturally occurring amino acids.

Suitable amino acids are amino acid pairs where one amino acid contains a free amino and another contains a free carboxyl. Examples of amino acids containing an R-group carboxyl moiety include naturally occurring amino acids such as aspartic acid and glutamic acid, and non-naturally occurring amino acids such as 2-aminohexanedioic acid and 2-aminoheptanedioic acid. Examples of amino acids containing an R-group amino moiety include naturally occurring amino acids such as lysine and ornithine, and non-naturally occurring amino acids such as diaminobutyric acid and diaminopropanoic acid.

Cyclization in an (i, i + 3), (i, i + 4) and (i, i + 7) spaced side chain to a side chain lactam bridge stabilizes turn conformations and helical segments. (Taylor *Biopolymers (Peptide Science)* 66:49-75, 2002.) A peptide can be modified to contain amino acids with appropriate groups (carboxyl and amino pairs) suitably spaced apart.

9.2. The Structures of HR Peptides Containing Lactam Bridges

In an embodiment of the present invention, the C-peptide consists of a sequence selected from the group consisting of: any of the sequences derived from SEQ ID NO:2, as disclosed in section 2;

that is (a) modified to contain one or more lactam bridges each of which is independently in an (i, i + 3), (i, i + 4) or (i, i +7) orientation; (b) optionally modified to contain one or more conservative substitutions; (c) optionally modified to contain a C-terminus deletion; and (d) optionally modified to contain a N-terminus deletion,

provided that the N-terminus, C-terminus, or both the N-terminus and C-terminus may contain a protecting group.

Examples of such HR2 peptides are partial or full sequence of SEQ ID NO:2. HR2 retro-inverso analogues can consist of amino acid residues from N to (N+M-1) of SEQ ID NO:33, wherein M is the length of the HR2 peptide, and N can be any integral number from 1 to (58-M).

Examples of peptides containing (i, 1+3), (i, i+4), or (i, i+7) lactam bridges are:

(i,i+3)

1. (Lys) (Asp)

P(Asp)VD(Lys)GDISGINASVVNIQKEIDRLNEVAKNLNES

(SEQ ID NO:43)

(i,i+4)

(Dab) (Glu)

2. DVDLG(Glu)ISG(Dab)NASVVNIQKEIDRLNEVAKNLNESL

(SEQ ID NO:44)

(i,i+7)

(Glu) (Orn)

3. VDLGDISGINA(Orn)VVNIQK(Glu)IDRLNEVAKNLNESLI

(SEQ ID NO:45)

10. The Production of the Inhibitors

The inhibitors can be produced by a variety of methods, including by means of recombinant DNA or RNA methods and / or known chemical synthetic methods. They can also be obtained from a library of peptides, such as a combinatorial peptide library. They may be capped or uncapped with certain amino

acids. Recombinant versions can contain helix capping residues on the N- terminus, the C-terminus or both. Possible capping residues for the N-terminus are amino acid T, D and S and for the C-terminus include amino acid G, R, H, N, and K.

The peptides may also contain a C-terminus cap (also referred to herein as a C-terminus "protecting group") or a N-terminus cap (also referred to herein as a N-terminus "protecting group"). A C-terminus protecting group is a modification to the C-terminal amino acid that reduces the reactivity of the carboxyl terminus under *in vivo* conditions. The carboxyl terminus is preferably modified at the α-carbonyl group. Examples of modification include chemical groups such as amide, methylamide, and ethylamide.

An N-terminus protecting group is a modification to the N-terminal amino acid that reduces the reactivity of the amino terminus under *in vivo* conditions. The N-terminal amino acid can be modified with groups such as acetyl, propionyl, succinyl, benzyl, benzyloxycarbonyl or *t*-butyloxycarbonyl.

10.1. The chemical synthesis of the peptides

According to an embodiment of the present invention, the peptides are produced using standard techniques including those involving step-wise chemical synthesis. Techniques for chemical synthesis of peptides are well known in the art. (See *e.g.,* Vincent, in *Peptide and Protein Drug Delivery*, New York, N.Y., Dekker, 1990; E. Atherton & R. C. Sheppard, *Solid Phase Peptide Synthesis. A Practical Approach.* IRL Press, Oxford, England, 1989; J. Jones, *The Chemical Synthesis of Peptides*, Oxford University Press, New York, 1991; M. Bodanszky & A. Bodanszky, *The Practice of Peptide Synthesis*, 2nd ed., Springer-Verlag, Berlin Heidelberg, 1994; Fields, G., Ed., *Solid-Phase Peptide Synthesis*. Methods Enzymol. 289, Academic press, New York, 1997).

Tryptophan analogs can be produced and incorporated into a peptide taking into account well known techniques. Different tryptophan analogs can be obtained from commercial sources such as RSP Amino Acid Analogues Inc., Bachem, and Sigma; or based on techniques such as those described by Huber *et al., Tetrahedron Letters 43*:6729-6733, 2002, Fredrickson *et al., European Journal of Pharmacology 458*(1-2):111-118, 2003, Mohammadi *et al., Biochemistry 40(34)*:10273-10283, 2001, Rajh *et al., International Journal of Peptide & Protein Research 14(1)*:68-79, 1979, and Cady *et al., Archives of Biochemistry and Biophysics 291(2)*:326-333, 1991.

Examples of techniques that can be used to produce and study peptides containing lactam bridges are provided by Taylor, *Biopolymers (Peptide Science) 66*:49-75, 2002 and Judice *et al., Proc. Natl. Acad. Sci. USA 94*:13426-13430, 1997.

The retro-inverso peptides of the present invention may be chemically synthesized, using the same methods utilized for the synthesis of normal peptides. See, e.g., *J. Biol. Chem.* 270:11921-11926

(1995), Briand et al., *Proc. Natl. Acad. Sci. U.S.A.* 94:12545-12550 (1997), Fletcher & Campbell, *Chem. Rev.* 98:763-795(1998), Levi *et al.*, *Res. Human Retroviruses* 16:59-65 (2000); Tayor *et al.*, 2000; Pescarolo *et al.*, *FASEB J.* 15:31-33 (2000); Chen *et al.*, *J. Med. Chem.* 45 1624-1632 (2002); D'Ursi *et al.*, *J. Med. Chem.* 46 1807-1810 (2003)

10.2 The production of Five-Helix proteins with recombinant DNA technologies

The present invention further relates to DNA encoding Five-Helix; methods of producing Five-Helix. Five-Helix protein can be produced by a variety of methods. For example, it can be produced from a larger protein, such as 6-Helix, by enzymatic (trypsin) digestion. Alternatively, it can be produced, using known methods and expression systems, by expressing Five-Helix protein-encoding DNA, which can be a single DNA that encodes the entire Five-Helix protein or two or more DNA "units", each of which encodes a portion (e.g., one or more HR1 component, one or more HR2 components) of Five-Helix protein.

The yield of expression and purification of Five-Helix can be significantly improved by direct expression of the Five-Helix gene in an appropriate host cell, such as *E. coli*. In this approach, the Five-Helix gene encodes the residues present in the final Five-Helix protein. A C-terminal His-tag can be attached to facilitate purification (with or without a protease cleavage site to later remove the tag). The protein can then be used directly without the proteolytic cleavage and unfolding steps required for producing Five-Helix starting from Six-Helix.

This Five-Helix molecule may be expressed as a folded active molecule, allowing its use in biological selections or screens for optimizing its properties. Alternatively, protein synthetic methods can be used to produce Five-Helix protein. The HR1 and HR2 components of Five-Helix can be joined covalently (such as by means of a linker of at least one (one or more) amino acid residues) or by other means which results in formation of a protein which is stable under physiological conditions and is correctly folded such that the remaining surface of Five Helix is presented so that it is available to bind or interact with coronavirus HR2. In the embodiments in which there are three HR1 components and more than two (but less than three complete) HR2 components, the components can be similarly joined.

Five-Helix can be assessed to determine if it acts on the virus before it enters the cell. Five-Helix should be soluble under physiological conditions and should also be stable under physiological conditions.

It should also be possible to generate 5-Helix variants with an increased molecular weight (by oligomerization or tethering to a large protein) to reduce the rate of kidney clearance. In addition, Five-Helix dimers can be made by disulfide crosslinking, to produce a molecule filtered to a lesser extent than the Five-Helix "monomer". Thus, it is reasonable to expect that dimers might have an enhanced

bioavailability when compared to that of the C-peptides. Bioavailability of Five-Helix can be affected (enhanced) by producing variants in nonbacterial cells, in which the protein would be glycosylated as it is produced. Alternatively, glycosylation sites can be engineered into Five-Helix.

Another embodiment of this invention is a complex of Five-Helix tethered (with an appropriate linker, which can be made of amino acid residues, or can be a chemical or synthetic linker) to a component, such as a ligand (protein or small molecule) that binds coronavirus envelope protein. The complex is useful as a therapeutic agent, because the ligand will increase the effective concentration of the tethered Five-Helix moiety in the vicinity of the coronavirus envelope protein.

11. Utilities of the Coronavirus Inhibitors

The present invention relates to methods in which the peptides and Five-Helix proteins are used. The peptides and Five-Helix are useful as therapeutic agents or drugs to prevent coronavirus infection or reduce the extent to which it occurs; as a reagent for identifying (screening for) or designing other therapeutics or prophylactics effective against coronavirus infection.

In one embodiment of the present invention, the peptides and Five-Helix are used to reduce coronavirus infection in an individual. In this embodiment, the peptides or Five-Helix is administered to an individual in sufficient quantity to reduce (totally or partially) infection of the individual's cells. That is, a dose of the peptides or Five-Helix sufficient to reduce coronavirus infection (an effective dose) is administered in such a manner (e.g., by injection, topical administration, intranasal route, intravenous route) that it inhibits (totally or partially) coronavirus entry into cells. The peptides or Five-Helix can be administered to an individual who is infected, to reduce further infection, or to an uninfected individual to prevent infection or reduce the extent to which infection occurs. The Five-Helix can also be administered via expression of Five-Helix-encoding DNA in appropriate host cells or vectors.

The present invention provides a method of inhibiting infection of human cells by coronavirus in an individual, comprising administering to the individual a pharmaceutical composition comprising the retro-inverso analogue of a SARS-CoV HR peptide in sufficient quantity to inhibit infection of human cells by the SARS virus, and a pharmaceutical acceptable carrier, wherein infection of human cells is inhibited.

11.1 Administration

HR-peptide coronavirus inhibitors can be formulated and administered to a subject using the guidance provided herein along with techniques well known in the art. The preferred route of administration ensures that an effective amount of compound reaches the target. Guidelines for

pharmaceutical administration in general are provided in, for example, *Remington's Pharm Sciences 18th Edition*, Ed. Gennaro, Mack Publishing, 1990, and *Modern Pharmaceutics 2* Banker and Rhodes, Marcel Dekker, Inc., 1990.

HR-peptide coronavirus inhibitors can be prepared as acidic or basic salts. Pharm acceptable salts (in the form of water- or oil-soluble or dispersible products) include conve toxic salts or the quaternary ammonium salts that are formed, *e.g.*, from inorganic or orga bases. Examples of such salts include acid addition salts such as acetate, adipate, alginate, benzoate, benzenesulfonate, bisulfate, butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucohep glycerophosphate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hyd hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nico pamoate, pectinate, persulfate, 3-phenylpropionate, picrate, pivalate, propionate, succinate thiocyanate, tosylate, and undecanoate; and base salts such as ammonium salts, alkali met sodium and potassium salts, alkaline earth metal salts such as calcium and magnesium sal organic bases such as dicyclohexylamine salts, N-methyl-D-glucamine, and salts with am as arginine and lysine.

HR-peptide coronavirus inhibitors can be administered using different routes such intravenous route, an intraperitoneal route, a subcutaneous route, a topical route, an intran or an intranasal route. When administered by injection, the injectable solution or suspens formulated using suitable non-toxic, parenterally-acceptable diluents or solvents, such as or isotonic sodium chloride solution, or suitable dispersing or wetting and suspending age sterile, bland, fixed oils, including synthetic mono- or diglycerides, and fatty acids, inclu

Pharmaceutically acceptable carriers facilitate storage and administration of a HF patient. Pharmaceutically acceptable carriers may contain different components such as a water for injection, normal saline or phosphate buffered saline, sucrose, histidine, salts ar

Suitable dosing regimens are preferably determined taking into account factors w art including type of subject being dosed; age, weight, sex and medical condition of the s of administration; the renal and hepatic function of the subject; the desired effect; and the compound employed.

Optimal precision in achieving concentrations of drug within the range that yield without toxicity requires a regimen based on the kinetics of the drug's availability to targ involves a consideration of the distribution, equilibrium, and elimination of a drug. The subject is expected to be between 0.01 and 1,000 mg per subject per day.

HR-peptide coronavirus inhibitors can be provided in kit. Such a kit typically contains an active compound in dosage forms for administration. A dosage form contains a sufficient amount of active compound such that a beneficial effect can be obtained when administered to a patient during regular intervals, such as 1 to 6 times a day, during the course of 1 or more days. Preferably, a kit contains instructions indicating the use of the dosage form for treating coronavirus and the amount of dosage form to be taken over a specified time period.

11.2. Immunogens

HR-peptides providing appropriate epitopes may be used as an immunogen for anti-coronavirus antibody production. The immunogen can be used, for example, as a vaccine component or to produce monoclonal or polyclonal antibodies that are subsequently isolated and used to target the coronavirus Spike protein. In an embodiment, the invention relates to a method of eliciting an immune response to coronavirus in an individual, comprising introducing, by an appropriate route, a composition comprising Five-Helix and a physiologically acceptable carrier, in a dose sufficient to elicit the immune response in the individual. Vaccines comprising the peptides or Five-Helix (or a variant or portion thereof) in a physiologically acceptable carrier are the subject of this invention.

Immunogens can be formulated as a vaccine or used for antibody production and isolation taking into account the guidance provided herein along with techniques well known in the art. Techniques for producing and using antibodies are well known in the art. Examples of such techniques are described in Ausubel, *Current Protocols in Molecular Biology,* John Wiley, 1987-1998, Harlow *et al., Antibodies, A Laboratory Manual,* Cold Spring Harbor Laboratory, 1988, and Kohler *et al., Nature 256*:495-497, 1975.

Guidelines for pharmaceutical vaccine administration in general are provided in, for example, *Vaccines* Eds. Plotkin and Orenstein, W.B. Sanders Company, 1999; *Remington's Pharmaceutical Sciences 20th Edition*, Ed. Gennaro, Mack Publishing, 2000; and *Modern Pharmaceutics 2nd Edition*, Eds. Banker and Rhodes, Marcel Dekker, Inc., 1990.

A vaccine can be administered in conjunction with an adjuvant. Adjuvants are substances that can assist an immunogen in producing an immune response. Adjuvants can function by different mechanisms such as one or more of the following: increasing the antigen biologic or immunologic half-life; improving antigen delivery to antigen-presenting cells; improving antigen processing and presentation by antigen-presenting cells; and inducing production of immunomodulatory cytokines. (Vogel, *Clinical Infectious Diseases 30* (suppl. 3):S266-270, 2000.)

A variety of different types of adjuvants can be employed to assist in the production of an immune response. Examples of particular adjuvants include aluminum hydroxide, aluminum phosphate, other salts of aluminum, calcium phosphate, DNA CpG motifs, monophosphoryl lipid A, cholera toxin, *E.*

coli heat-labile toxin, pertussis toxin, muramyl dipeptide, Freund's incomplete adjuvant, MF59, SAF, immunostimulatory complexes, liposomes, biodegradable microspheres, saponins, nonionic block copolymers, muramyl peptide analogues, polyphsophazene, synthetic polynucleotides, IFN-γ, IL-2 and IL-12. (Vogel *Clinical Infectious Diseases 30*(suppl 3):S266-270, 2000, Klein *et al., Journal of Pharmaceutical Sciences 89*, 311-321, 2000.)

Vaccines can be administered by different routes such as subcutaneous, intramuscular, or mucosal. Subcutaneous and intramuscular administration can be performed using, for example, needles or jet-injectors.

Suitable dosing regimens are preferably determined taking into account factors well known in the art including age, weight, sex and medical condition of the patient; the route of administration; the desired effect; and the particular compound employed. The immunogen can be used in multi-dose vaccine formats. It is expected that a dose would consist of the range of 1 μg to 1.0 mg total polypeptide, in an embodiment of the present invention the range is 0.1 mg to 1.0 mg.

The timing of doses depends upon factors well known in the art. After the initial administration one or more booster doses may subsequently be administered to maintain or boost antibody titers. An example of a dosing regime would be day 1, 1 month, a third dose at either 4, 6 or 12 months, and additional booster doses at distant times as needed.

The retro-inverso peptides can also be used as vaccines against SARS. Several studies have documented that the retro-inverso analogs of linear peptide epitopes can be useful as vaccines (Guichard *et al., Proc. Natl. Acad. Sci. U.S.A.* 91:9765-9769 (1994); Benkirane *et al., J. Biol. Chem.* 270:11921-11926 (1995); Briand *et al., Proc. Natl. Acad. Sci. U.S.A.* 94:12545-12550 (1997); Meziere *et al.*, 1997; Muller & Briand, *Res. Immunol.* 149:55-57 (1998)). More specifically it was shown for Foot-and-Mouse disease virus (FMDV) that (i) antibodies raised against the natural epitope recognize the retro-inverso peptide; (ii) the retro-inverso peptide is able to generate in rabbits an immune response of much higher titer, and longer duration, than the corresponding linear peptide; (iii) the antibodies generated by immunization with the retro-inverso peptide are cross reactive with FMDV viral particles, and (iv) the immune response generated in guinea pigs by immunization with the retro-inverso peptide is protective against FMDV challenge (Briand *et al., Proc. Natl. Acad. Sci. U.S.A.* 94:12545-12550 (1997)).

Therefore retro-inverso peptides, corresponding to the sequence of the Heptad Repeats 1 and 2 of the SARS coronavirus, can be used, either as such or, more desirably, conjugated to a protein carrier like OMPC, for the induction of a protective immune response against the virus.

11.3. Other Utilities

In a specific embodiment, the invention relates to a method of identifying a compound or molecule that binds the peptides or Five-Helix and inhibits coronavirus infection of mammalian cells. The compound or molecule to be assessed is referred to as a candidate inhibitor. The method comprises combining a candidate inhibitor and the peptides or Five-Helix under appropriate binding conditions, and determining and selecting the compound or molecule that binds the peptides or Five-Helix. The method optionally further comprises determining and selecting the selected compound or molecule that inhibits coronavirus infection of mammalian (e.g., human) cells, such as in a cell-based assay. Such a compound or molecule may be used to inhibit (totally or partially) coronavirus infection of human or non-human animals.

Other embodiments are within the following claims. While several embodiments have been shown and described, various modifications may be made without departing from the spirit and scope of the present invention.

EXAMPLES

Examples are provided below to further illustrate different features of the present invention. The examples also illustrate useful methodology for practicing the invention. These examples do not limit the claimed invention.

Example 1: The Assays Used to Test the Peptide Activity

The identification of SARS coronavirus HR1 and HR2 sequences provides substrates that can be employed to evaluate the ability of compound to inhibit HR1 and HR2 interactions. Compounds that can be evaluated include C-peptides and N-peptides.

Different types of assay formats can be employed to measure the ability of a compound to inhibit HR1 and HR2 interactions. Detectable labels on either or both HR1 and HR2 can be used to help measure HR1 and HR2 interactions. Examples of different types of labels include fluorescent labels, radioactive labels, and donor-acceptor pairs (*e.g.*, FRET labels).

Peptide activity can also be measured by providing a peptide to a cell infected with the coronavirus. Using techniques well known in the art, different types of assays and endpoints can be measured to evaluate peptide activity. For example, an assay can be performed to measure a compound's ability to prevent virus infection and cell-to-cell spread over a 72-hour incubation; where antiviral activity is assayed by measuring retention of the cells' ability to phagocytose neutral red. An example of such as assay (based on USAMRIID assay) is as follows:

1) Incubate cells with different concentrations of test peptide (may be done in triplicate). The different concentrations may be serial dilutions and should include a control with no peptide. Peptide dilutions can be performed in a suitable buffer (*e.g.*, EMEM, 1% fetal bovine serum, 1.0% L-glutamine (100 X), 1.0% HEPES, 0.1% Fumigzone (250 µg/ 20 ml), 0.1% Gentamicin (50 mg/ml)) added to a 96-well microtiter plate of confluent Vero 76 cells (VERO 76, ATCC number CRL-1587). At different drug concentrations wells are inoculated with SARS associated coronavirus (*e.g.*, MOI of 0.01) or are not infected.

2) Plates are incubated at 37°C, 5% CO_2 and examined daily until a desired cytophathic effect is seen in untreated cells (*e.g.*, 4 +CPE). Cell viability can be assessed by measuring neutral red uptake. For example, neutral red can be added to give a final concentration of 0.22 mg/ml and the cells are incubated at 37°C for 1.5 hours. The medium is then removed; wells are rinsed three times with phosphate buffered saline and fixed in 10% buffered formalin for 15 minutes. Retained neutral red is solubilized with 100 µl of a 50% ethanol, 50% 0.01 M $NH_4H_2PO_4$ (pH 3.5). Plates can then be rocked for 30 minutes at 150 RPM to liberate neutral red and the optical density (at 450 nm) can be measured with a plate reading spectrometer.

Other embodiments are within the following claims. While several embodiments have been shown and described, various modifications may be made without departing from the spirit and scope of the present invention.

Example 2: An assay based on the pseudotyping of HIV cores with SARS-CoV Spike protein.

The present invention also provides a SARS-spike dependent entry assay useful for screening inhibitors of SARS-CoV entry and fusion. In this assay, cells transiently or stably expressing angiotensin converting enzyme-2 (ACE-2) are infected with HIV-1 Luciferase reporter viruses pseudotyped with SARS-CoV Spike protein.

HIV DNA that has a deletion in its envelope sequence and containing firefly luciferase in place of the Nef gene is co-transfected with a mammalian expression vector encoding codon optimized SARS-CoV Spike protein. A plasmid DNA encoding the NL4-3 viral clone, which contains a deletion in its envelope sequence and containing firefly luciferase in place of the Nef gene is co-transfected in 293T cells (a human embryonic kidney cell line stably expressing the Simian Virus 40 Large T antigen) with a mammalian expression vector encoding a codon optimized SARS-CoV Spike protein (See, e.g., Simmons, G., et al., *PNAS* 101:4240-5 (2004)).

The transfected cells produce in their supernatants HIV cores that are pseudotyped with SARS Spike protein. These reporter viruses are capable of entering 293T cells transiently or stably transfected

with expression vectors encoding the angiotensin converting enzyme-2 (ACE-2), a cellular receptor for SARS-CoV (Li, W., et al., *Nature* 426:450-4 (2003); Wong, S.K., et al., *Journal of Biological Chemistry* 279:3197-201 (2004)).

Infection is monitored by measuring Firefly luciferase activity in cell lysates at 48-72h post-infection. The system offers a dynamic range (30-100 fold above infection with particles lacking virus envelopes). Entry through the ACE-2 receptor has been validated in both in transiently and stably transfected cells by demonstrating that particle entry is blocked in a dose-dependent fashion with antibodies directed to ACE-2.